Kidney Aminopeptidase A and Hypertension, Part II
Effects of Angiotensin II

Lijun Song, Dennis P. Healy

Abstract—Aminopeptidase A (APA) is the principal enzyme that metabolizes angiotensin II (Ang II) to angiotensin III. Previously, we showed that kidney APA was elevated in spontaneously hypertensive rats and was reduced after angiotensin-converting enzyme inhibition. In the present study, we sought to determine whether kidney APA expression was altered after chronically elevated Ang II, either exogenously delivered via osmotic minipumps or endogenously produced in two-kidney, one clip (2K1C) hypertensive rats. Ang II (200 ng \( \cdot \) kg\(^{-1} \cdot \) min\(^{-1} \)) was infused subcutaneously for 1 or 2 weeks by osmotic minipumps, and 2K1C rats were tested 4 weeks after unilateral renal artery clipping. Blood pressure was not significantly elevated in the Ang II–infused animals but was significantly increased at 3 and 4 weeks in the 2K1C animals. APA was significantly elevated approximately 2-fold in kidney cortical membranes from Ang II–infused animals but was decreased 45% in the clipped kidney and 18% in the nonclipped kidneys from 2K1C animals. Isolated glomeruli from Ang II–infused animals and the nonclipped kidneys from 2K1C animals had markedly higher APA activity and immunoreactivity. Likewise, histochemical and immunohistochemical studies indicated that APA levels were increased in glomeruli from angiotensin-infused animals and in both nonclipped and clipped kidneys from 2K1C animals. In contrast, tubular APA was decreased in tubular elements from 2K1C animals, most markedly in the clipped kidneys. Thus, despite the increase in glomerular APA expression in kidneys from 2K1C animals, the decrease in tubular APA expression is more extensive and accounts for the measured reduction in total APA in cortical homogenates. Because clipped kidneys are not exposed to high blood pressure, these results suggest that glomerular APA expression is positively regulated and tubular APA negatively regulated by Ang II. These results further suggest that changes in kidney APA expression could influence the progression of angiotensin-dependent hypertension.

Key Words: aminopeptidases • angiotensin II • angiotensin III • kidney glomerulus • proximal renal tubules

The therapeutic efficacy of angiotensin-converting enzyme (ACE) inhibitors and angiotensin type 1 (AT\(_1\)) receptor antagonists in hypertension and other cardiovascular diseases\(^{1,2}\) has led to the widely held view that elevated activity of the renin-angiotensin system (RAS) gives rise to these conditions. Support of this concept has been provided by studies in which overexpression of components of the RAS in transgenic animals results in hypertension.\(^{3,4}\) However, whereas an impairment in angiotensin degradation coupled with normal synthesis, ie, a “decreased degradation” model, could theoretically increase angiotensin levels and would be indistinguishable from the “elevated synthesis” model in that both would be effectively controlled by ACE inhibition or angiotensin type 1 antagonists, the role that angiotensin degradation plays in the progression of angiotensin-dependent cardiovascular disease has been largely unexplored. The principal route for degradation of angiotensin II (Ang II) in the circulation is aminoterminally directed by a series of aminopeptidases.\(^{5,6}\) The first step in the hydrolysis of Ang II to des-Asp\(^1\)-Ang II (angiotensin III) is mediated by aminopeptidase A ([APA], glutamyl aminopeptidase EC 3.4.11.7).\(^{7-9}\)

Early studies failed to detect any change in plasma levels of APA from essential and renal hypertensive patients compared with normotensive patients,\(^{10}\) leading to the view that angiotensinases are unregulated. It has been widely shown, however, that measurement of plasma components of the RAS are not reliable indicators of changes in activity within tissues.\(^{3,11,12}\) Among the various vascular beds, Ang II is most rapidly extracted from the renal circulation.\(^{13}\) APA is highly expressed within the kidney, with the highest levels being found within proximal tubules and glomeruli.\(^{14}\) Various lines of evidence conclude that renal dysfunction plays a prominent role in the development and maintenance of hypertension.\(^{15}\) In spontaneously hypertensive rats (SHR), transplantation of SHR kidneys into normotensive animals produces hypertension in the recipients.\(^{16}\) Likewise, young SHR are hypersensitive to intrarenal injections of Ang II.\(^{17,18}\) Whereas one might predict that a decrease in expression of APA in SHR kidneys may contribute to the development of hyper-
tension in this model, we determined that kidney APA was elevated in prehypertensive, developing, and established phases of hypertension.\textsuperscript{19} Further, we found that ACE inhibition reduced kidney APA activity, suggesting that APA expression may be positively regulated by Ang II. The possibility is supported by evidence that intrarenal Ang II is elevated in young SHR.\textsuperscript{20} Thus, upregulation of APA by Ang II may be a homeostatic mechanism by which elevated Ang II speeds its degradation, and this mechanism may act in parallel with the negative feedback inhibition of renin release by Ang II to reduce the levels of Ang II.\textsuperscript{19} Whereas the upregulation of APA does not prevent development of hypertension in SHR, failure of APA to upregulate or a deficiency in APA expression would be expected to speed development of this form of hypertension. Indeed, intravenous administration of purified APA reduces blood pressure in SHR in a dose-dependent manner,\textsuperscript{21} showing that modulating APA levels may have important consequences with regard to blood pressure regulation.

Although ACE inhibition reduced APA activity in SHR kidney, blood pressure was also reduced by this treatment, leaving open the possibility that the reduction was secondary to the fall in blood pressure. In the present study, we sought to determine the direct effects of Ang II on APA expression but by minimizing the indirect effects due to elevation of blood pressure. We used 2 approaches. The first was to infuse chronically, via osmotic minipumps, nonpressor doses of Ang II. The second approach used two-kidney, one clip (2K1C) Goldblatt hypertensive rats, a renin-dependent model of hypertension.\textsuperscript{22} Immediately after unilateral placement of a silver clip to restrict blood flow, plasma renin becomes elevated because of increased synthesis and release of renin by the ischemic kidney. Plasma renin remains elevated for a period of several weeks after which, over the next 2 to 5 weeks, renin levels return to normal as hypertension develops and perfusion to the clipped kidney returns to normal. The nonclipped kidney is thus exposed to high blood pressure and high Ang II levels via the systemic circulation, whereas the clipped kidney is exposed to high Ang II levels and low to normal blood pressure. The advantage of this model is that it allows differentiation between direct effects of Ang II versus effects secondary to elevations in blood pressure.

**Methods**

**General**

Male Sprague-Dawley rats (175 to 225 g. Charles River Breeding Laboratories, Wilmington, MA) were housed on a 12-hour-light/12-hour-dark schedule and allowed free access to food and water. Rats (n = 42) were left nephrectomized under ketamine/xylazine anesthesia. An osmotic minipump (Model 1001 or Model 2002, Alza Corp) containing either Ang II (Sigma Chemical) or vehicle was implanted subcutaneously in the scapular region at the dorsum of the neck. Ang II was delivered at a rate of 200 ng kg\textsuperscript{−1} h\textsuperscript{−1} per minute for a period of 1 or 2 weeks. Six or 12 days after surgery, blood pressures were measured by a tail-cuff sphygmomanometer (ITC Inc). Three to 5 measurements from a single animal were averaged. One or 2 weeks after surgery, the rats were killed by decapitation, blood was collected into heparinized tubes, and the remaining kidney was removed and frozen on dry ice. Kidney glomeruli were isolated as previously described\textsuperscript{14} from kidneys pooled from 6 animals.

For studies with 2K1C Goldblatt hypertensive rats, a silver clip (ID 0.2 mm) was placed over the left renal artery of rats under pentobarbital anesthesia (50 mg/kg, IP). Sham-operated controls simply had the left renal artery exposed. Blood pressures were monitored weekly from both the 2K1C and sham-operated groups for 4 successive weeks by tail-cuff plethysmography (ITC Inc). Four weeks after surgery, the rats were decapitated and truncal blood was collected into heparinized tubes. The left (clipped or normal) and right kidneys were processed for histochemistry, immunohistochemistry, and measurement of APA activity. Glomeruli were isolated as described previously\textsuperscript{14} from kidneys pooled from 6 animals.

**Enzymatic Assay**

APA enzyme activity from kidney membranes was measured as previously described with use of \(\alpha\)-glutamyl-2-naphthylamide (Bachem Bioscience, Philadelphia, PA) as substrate.\textsuperscript{14} Specific activities were expressed as \(\mu\text{mol substrate} \cdot \text{hydrolyzed mg} \cdot \text{tissue}^{-1} \cdot \text{h}^{-1}\). Kidney samples were homogenized with a Polytron at 4°C for 20 seconds in 0.05 mol/L Tris-HCL buffer, pH 7.5. A low-speed spin (500g) was used to remove cellular debris. The supernatant was then centrifuged at 40 000g to collect crude membranes that were used for enzymatic assay and immunoblotting.

**Immunoblotting**

Immunoblotting was conducted as previously reported with some modifications.\textsuperscript{15} Crude kidney membranes were prepared by boiling for 5 minutes in buffer containing 60 mmol/L Tris-HCL, 2% SDS, 100 mmol/L DTT, and 0.01% Coomassie brilliant blue and were centrifuged in a Microfuge for 10 minutes. The resulting supernatants of kidney protein were separated by 10% SDS-PAGE (kidney samples 40 µg, isolated glomeruli 20 µg) and then transferred to a nylon membrane (Immobilon) in the presence of a transfer buffer containing 25 mmol/L Triza base, 192 mmol/L glycine, and 15% methanol at 70 V for 1 hour. The membrane was treated with a blocking buffer containing 5% nonfat dry milk and 0.02% NaN\textsubscript{3} with agitation at 37°C for 1 hour. The blocked membrane was washed twice with PBS for 5 minutes and then was incubated with primary APA antiserum (1:3000) at 4°C overnight. The membrane was washed 4 times with PBS for 5 minutes and then was incubated with primary APA antiserum (1:3000) at 4°C overnight. The membrane was washed 4 times with PBS for 5 minutes and then was incubated with peroxidase-labeled goat secondary antibody against rabbit IgG at 37°C with agitation for 3 hours. The membrane was washed 4 times with PBS for 5 minutes and was incubated with 10 mL 0.05 mol/L Tris-HCl (pH 7.6) containing 6 mg diaminobenzidine and 10 µL 30% H\textsubscript{2}O\textsubscript{2} for 5 minutes. The membrane was then washed with PBS and was dried. For quantification, samples were visualized by luminescence (ECL Western blotting, Amersham International). The labeling intensity of the bands was performed by an Agfa Arcus flatbed scanner interfaced to a computer running ImageQuant (Molecular Dynamics) densitometry software.

**Immunohistochemistry**

The immunohistochemical staining of APA with fluorescein-labeled avidin in rat kidney was performed according to methods previously described.\textsuperscript{14} Briefly, frozen sections were sectioned by cryostat and 20-μm sections were collected onto cold slides coated with silane. Sections were fixed for 5 minutes in 3% paraformaldehyde, were rinsed twice in PBS for 2 minutes, and were passed sequentially through H\textsubscript{2}O, 50% ethyl alcohol (ETOH), 70% ETOH, 95% ETOH, and 100% ETOH for 1 minute each. The vacuum-dried sections were then preincubated with 2% normal goat serum in PBS containing 0.3% Triton X-100 RT for 30 minutes followed by incubation with primary APA antiserum (1:500 dilution) in PBS containing 0.1% Triton X-100 and 0.1% BSA at 4°C overnight. Sections were then rinsed 4 times with PBS for 5 minutes and were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) in PBS-Triton X-100 (1:222 dilution) at room temperature for 45 minutes. The sections were washed 4 times with PBS for 5 minutes and were incubated with fluorescein-avidin reagent/PBS-Triton X-100 (1:100 dilution) at room temperature for 1 hour. The slides were rinsed 4 times with PBS for 5 minutes and were air dried. The immunofluo-
Results

Blood Pressure
Subcutaneous infusion of Ang II at a rate of 200 ng·kg⁻¹·h⁻¹ for 1 or 2 weeks did not result in any significant difference in blood pressure, although blood pressures were slightly increased in the Ang II–infused groups compared with vehicle-infused animals (Table 1). Blood pressure increased progressively in the 2K1C and sham-operated control rats through the 4 weeks of the study (Table 1). Both systolic and mean blood pressure of 2K1C rats were higher than that of control rats, although blood pressures were slightly increased in both the 1- and 2-week Ang II–infused groups of animals compared with vehicle-infused controls and 2K1C animals and their sham-operated controls via a tail-cuff monitor (IITC). Three readings were averaged for each animal per session. Values are expressed as mean±SE. Number of animals is in parentheses. * P<0.05, Student’s t test versus control.

Enzyme Activity
APA enzyme activity in kidney membranes prepared from both 1- and 2-week Ang II infusion groups was significantly higher than in control rats (Table 2). Plasma APA activity was unchanged. Glomeruli isolated from 1- or 2-week Ang II–infused rats had increased APA activity that was more than 2-fold compared with control rats (Table 2). In contrast, APA enzyme activity was significantly lower (P<0.01) in cortical membranes from the clipped kidneys compared with sham-operated control kidneys (Table 2). The clipped kidney also had lower APA activity compared with the nonclipped kidney (P<0.05). Plasma APA activity was not significantly different between the 2K1C group and sham-operated controls. APA activity was increased 1.7-fold, however, within iso-

Immunoblots
Immunoblot analysis of kidney membranes from Ang II–infused and vehicle-infused control rats revealed labeling of an APA at ≈140 kd as previously described. There was a 1.7-fold increase (P<0.01) in the labeling of the APA band from Ang II–infused rats compared with vehicle-infused rats (Figure 1). Glomeruli isolated from the Ang II–infused animals also had elevated levels of APA (Figure 2). Densitometric analysis of the labeled bands indicated that APA was increased approximately 10.2-fold in isolated glomeruli from the 1-week Ang II–infused animals and 4-fold in the 2-week infused animals compared with glomeruli from control animals. Immunoblot analysis of isolated glomeruli from nonclipped and sham-operated kidneys indicated that the APA immunoreactivity was higher in nonclipped kidney glomeruli than from controls (Figure 2). Densitometric analysis of the labeled bands indicated a 33% increase in labeling from the nonclipped kidney compared with sham-operated controls.

APA Histochemistry
Histochemical staining of APA in kidney sections revealed that APA activity was principally elevated in glomeruli of both the 1- and 2-week Ang II–infused groups of animals compared with control animals (Figure 3). There was no clear difference in tubular APA activity between groups.

APA Activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ang II Infusion</th>
<th>2K1C</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week</td>
<td>2 weeks</td>
<td>Control</td>
</tr>
<tr>
<td>Kidney ctx (μmol/mg/h)</td>
<td>113±22.7 (9)</td>
<td>84.7±5.8 (9)</td>
<td>40.4±3.9 (12)</td>
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<tr>
<td>Isol. Glom. (μmol/mg/h)</td>
<td>38.1</td>
<td>44.4</td>
<td>14.8</td>
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<tr>
<td>Plasma (μmol/ml)</td>
<td>2.4±0.1 (9)</td>
<td>2.9±0.2 (9)</td>
<td>2.7±0.5 (12)</td>
</tr>
</tbody>
</table>

Uninephrectomized rats were infused subcutaneously with Ang II (200 ng/kg/min) for 1 or 2 weeks and compared with vehicle-infused uninephrectomized controls. Clipped and nonclipped kidneys from 2K1C rats were compared with sham-operated controls. Data are expressed as mean±SE. * P<0.05; ** P<0.01; ANOVA and Dunnett’s multiple comparison test. Number of animals used for each measurement is in parentheses. Isolated glomeruli were collected from 6 kidneys pooled from separate animals.
APA histochemical activity within kidney sections from 2K1C sham-operated controls was primarily localized to tubule elements and glomeruli (Figure 4A and 4B). Nonclipped kidneys had less tubular staining and an increase in glomerular APA histochemical activity (Figure 4C and 4D). Whereas the increase in glomerular APA histochemical activity was uniform, the reduction in tubular APA activity was somewhat segmental with some areas being less affected. The most marked differences in APA histochemical staining were within the clipped kidneys (Figure 4E and 4F). Glomerular APA activity was much greater and tubular activity greatly diminished throughout the kidney. Histological examination of kidney sections from all 3 kidney types was unremarkable except that the clipped kidneys had some degree of tubulointerstitial fibrosis and tubular atrophy.

APA Immunohistochemistry

Immunohistochemical staining of APA in kidney sections taken from 1- and 2-week Ang II–infused rats showed that APA was particularly increased in glomeruli from both groups but in particular within the 2-week group (Figure 5). In 2K1C animals, APA immunofluorescence was higher in the glomeruli from both nonclipped and clipped kidneys compared with that of sham-operated control rats (Figure 6). As noted in the Methods section, the immunofluorescent procedure that was used was not optimal for tubular localization, leaving a high autofluorescent background (Figure 6D). However, specific tubular staining was primarily of the apical lining, consistent with previous studies. The apical staining was diminished in sections from both clipped and nonclipped kidneys.

Discussion

These experiments were designed to test the relationship between levels of Ang II and expression of APA in rat kidney. Low-dose Ang II was infused for a period of 1 or 2 weeks or generated endogenously in 4-week 2K1C hypertensive rats, and kidney APA expression was examined. The 2K1C model is attractive in that intrarenal Ang II levels are increased in both clipped and nonclipped kidneys but only the nonclipped kidney experiences elevated blood pressure. There were similarities and differences with the results from these 2 models. The levels of APA in glomeruli were increased in both 1- and 2-week
Ang II–infused animals and in both clipped and nonclipped kidneys from 2K1C animals. The increase in glomerular APA in kidneys from each model suggests that the increase is a direct effect of Ang II and not secondary to the elevation of blood pressure. Tubular levels of APA, on the other hand, were not detectably different in the Ang II–infused animals but were decreased in kidneys from 2K1C animals, dramatically so in the clipped kidneys. It is not clear whether the differences in tubular expression of APA between the 2 models are related to the length of the experiment (2 versus 4 weeks) or to the levels of Ang II that are achieved, with Ang II levels certainly higher in the 2K1C animals than in Ang II–infused animals since the latter group was not hypertensive. Taken together, one interpretation of these results is that glomerular APA is positively regulated by Ang II and tubular APA is negatively regulated.

An increase in glomerular APA histochemical activity was also seen by Johnson et al.23 after infusion of pressor doses of Ang II. Here we showed that low-dose Ang II also upregulates glomerular APA activity, with the increase seeming to be because of increased levels of the enzyme, as seen both in immunoblots and immunohistochemically. Infusion of Ang II at doses that produce moderate hypertension have been shown to result in marked glomerular injury accompanied by increased deposition of matrix proteins.23,24 Indeed, renal injury due to elevated Ang II is known to play an important role in the progressive loss of renal function in diseases in which glomerulosclerosis is secondary.25 ACE inhibitors have been shown to delay the progression of glomerulosclerosis.26,27 APA is expressed both within mesangial cells and podocytes.14,28,29 Whereas upregulation of APA within glomeruli may play a protective role against the adverse effects that Ang II produces within glomeruli by decreasing the local concentration of Ang II, the current findings suggest that the increase in APA does not prevent the occurrence of glomerular injury. However, Wolf et al.30 recently showed that overexpression of APA blocked the mitogenic effects of Ang II on mesangial cells in vitro. Thus, upregulation of APA may indeed prevent or retard the development of glomerular sclerosis but may be limited from doing so by the capacity of the endogenous system. Conversely, failure of APA to upregulate in the presence of elevated intrarenal Ang II or a primary defect in APA could be potential risk factors for Ang II–mediated renal diseases.

The most surprising result from the present study was the decrease in APA activity in proximal tubules from 2K1C animals. Indeed, it is possible to speculate that the reduction in tubular APA may actually contribute to the maintenance of this form of hypertension. Ang II promotes sodium reabsorption from the proximal tubules.31–34 A reduction in APA activity within the proximal tubule, therefore, would facilitate the tubular effects of Ang II and enhance sodium and water reabsorption. Whereas total body sodium retention does not seem to be a prominent feature of 2K1C renovascular hypertension, the general view is that the clipped kidney retains sodium whereas the nonclipped kidney excretes sodium. However, in 2K1C hypertension, the nonclipped kidney contributes significantly to development of this form of hypertension by exhibiting an inappropriate natriuretic response to rising blood pressure.35,36 Whereas the increase in blood pressure may be expected to decrease tubular reabsorption, elevated activity of the RAS seems to counteract the increase in blood pressure such that tubular function in the nonclipped kidney is normal. The increased activity of the RAS in the nonclipped kidney is revealed when 2K1C animals are administered ACE inhibitors or Ang II receptor antagonists. These animals exhibit a marked natriuresis despite simultaneous reductions in blood pressure.37,38 Thus, it has been concluded that there is an Ang II–mediated enhancement in tubular reabsorption of sodium in nonclipped kidneys from 2K1C animals.37 The basis for the increase in Ang II–mediated tubular reabsorption in the nonclipped kidney seems to be elevated intrarenal content of Ang II.24,39 This finding seems paradoxical, inasmuch as renin levels are reduced in the nonclipped kidney.40 Although a number of explanations have been put forward,41–42 it is possible to speculate that the increase in kidney Ang II content may be related to a decrease in kidney APA. Thus, the reduction in tubular APA expression in nonclipped kidneys may contribute to the Ang II–mediated enhanced sodium reabsorption seen in the nonclipped kidney and, as such, may indirectly influence the progression of this form of renovascular hypertension.

Whereas there were marked changes in expression of APA within the kidney, there was no change in plasma levels of APA. The lack of change in plasma APA is consistent with early studies with renovascular hyperten-
sive patients in which no difference was seen. Indeed, this early report contributed to the generally held belief that angiotensinases are housekeeping enzymes. However, as shown numerous times, the level or activity of components of the RAS in plasma may or may not reflect the activity of the system within tissues. Moreover, the results presented here strongly suggest that APA can indeed be regulated by a physiological substrate, suggesting that regulation of Ang II degradation may play a role in regulation of activity of the RAS.

In summary, these studies indicate that animals exposed to increased levels of Ang II have elevated expression of APA in glomeruli regardless of whether the kidneys are exposed to elevated pressure. Upregulation of APA presumably plays a protective role against the adverse effects of Ang II in the development of glomerulosclerosis. In the clipped kidney from 2K1C animals in which renin secretion is elevated and Ang II is high, downregulation of APA within proximal tubules kidney presumably facilitates the tubular effects of Ang II on sodium reabsorption. In the nonclipped kidney, the same changes in cellular APA expression may have negative consequences. A reduction in APA would increase intrarenal Ang II levels by decreasing degradation of either systemically delivered or intrarenally formed Ang II. Such an increase in intrarenal Ang II would facilitate sodium retention by the nonclipped kidney and could contribute significantly to the development of 2K1C hypertension. The reduction in tubular APA may account, therefore, for the enhanced Ang II-mediated tubular sodium reabsorption that occurs in these kidneys and would tend to oppose the pressure-natriuretic effects that the elevated blood pressure has on renal function. These results also contribute to the growing body of evidence that APA can be regulated by Ang II and that degradation/conversion of Ang II to angiotensin III may represent another level at which activity of the RAS can be regulated.

Acknowledgments

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